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Introduction

Fundamental research clearly shows that the product of the c-myc oncogene is often expressed at elevated levels in a large number of breast cancers and is associated with poor prognosis, high-risk disease (for further detail, see our recent review (3). Indeed, Myc drives cell proliferation and can initiate as well as contribute to tumor development, as confirmed by both in vitro and in vivo models of breast cancer. The highly conserved regions of the Myc protein are thought to participate in protein:protein interactions. Importantly, blocking these sites can effectively inhibit Myc function and block breast carcinoma cell growth. Thus, Myc is a valid target for the development of novel therapeutics to inhibit mammary cells of malignant transformations in a specific and sensitive manner. This is the primary goal of this proposal.

To achieve our goal, we are using the novel Repressed Transactivator (RTA) assay developed in collaboration with our lab (1). The RTA is an in vivo functional assay that will enable Myc-binding proteins and inhibitors of Myc:protein interactions to be identified and characterized. This experimental tool is conceptually similar to the conventional "two-hybrid" technology but offers significant improvement. First, the RTA allows transactivator bait proteins like Myc, to be used in a two-hybrid approach to clone novel interactors. Second, the ability of the RTA to identify inhibitors of a given protein:protein interaction makes it particularly well suited for high throughput screening of protein:protein interactions and the identification of inhibitor compounds.

Objective/hypothesis

Our hypothesis is that Myc is a valid target for the development of novel therapeutics aimed at blocking essential Myc:protein complexes that drive the carcinogenic process of mammary epithelial cells.

Specific Aims

a. We will use the RTA to identify the Myc-binding domain of a recently identified Myc-interactor, TRRAP (2), and delineate the nature of a "Myc-signature sequence" with which other Myc-binding proteins can be identified by sequence databank analysis.
b. Clone and characterize novel Myc-binding protein using the RTA to screen for MycNTD interactors in cells derived from human breast carcinomas.
c. With these experimental tools in hand we will then use the RTA to identify specific inhibitors of these protein:protein interactions that will block Myc function and inhibit tumor cell proliferation.

Body

With support from the DOD, the research outlined in the original proposal has progressed in a steady and productive manner. To delineate the accomplishments, each task outlined in the Statement of Work is itemized below. The results of each task is summarized and the annual report in which the work the work was presented is provided.

Statement of Work

Task 1. To identify the Myc binding domain of TRRAP, a novel MycNTD-binding protein recently identified biochemically (months 1-18)

a. Completed, 2002. Overlapping fragments of TRRAP were cloned into the pBD prey vector using a PCR based approach and the sequence was verified as in-frame with the TUP-1 repressor domain.

b. Completed, 2002. The yeast were transformed with these TRRAP fusion proteins, and analyzed by western blot for equal expression of prey fusion proteins using our recently derived TUP-1 specific antibody.

c. Completed, 2002. It was established that the prey fusion proteins do not affect yeast viability in the presence or absence of 5-FOA.

d. Completed, 2002. The TRRAP fusion proteins were tested for their interaction with wildtype MycNTD bait proteins, include MbI and MBII deletion and point mutants as well as specificity controls for MycNTD interaction, including Gal4E2F, Gal4VP16, etc.

e. Completed, 2004. The region of TRRAP required for interaction with Myc was further refined using smaller fragments of TRRAP in the RTA (See Annual Report 2003). This resulted in the identification of a 336 amino acid fragment of the 3830 amino acid TRRAP protein that was sufficient to interact with the MycNTD (Fig 1). To further resolve the binding sites we employed smaller fragments of TRRAP and surveyed a panel of Myc NTD point mutants, but were unable to conclusively identify the key amino acid contact points. We then worked in collaboration with Dr. Cheryl Arrowsmith to express smaller fragments of the Myc-binding region of TRRAP in vitro to identify the smallest fragment that would interact with the MycNTD. This analysis showed that 160 amino acids of TRRAP are sufficient to interact with the smallest defined region of Myc essential for interaction (MBII) (Fig 1). Further analysis by circular dichroism confirms the interaction of the 160 amino acids and the MycNTD MBII region. However, many different approaches and attempts to scale-up the production of the polypeptides for Nuclear Magnetic Resonance (NMR) analysis and identify the points of interaction between TRRAP/Myc, have been tested and exhausted but have not yielded the required reagents to conduct NMR. Thus, the smallest region identified as essential for MycNTD MBII interaction is the 160 amino acids of TRRAP (Fig 1).

f. Completed, 2004. We have searched the sequence database for other polypeptides encoding this region and no hits were identified. This is not surprising given most interaction motifs are much smaller in size than 160 amino acids.

g. Not Applicable, 2004. If such proteins had been identified (see Task 1, part f above), their cDNA would have been cloned and tested for Myc interaction using the RTA. No proteins containing the 160 amino acid region were identified. Thus, this task is not applicable.

h. Not Applicable, 2004. No additional proteins were identified by searching for the 160 amino acid region in the protein database, thus further characterized was not warranted.

Task 2. To use the RTA to identify MycNTD-binding proteins that are essential for Myc to transform mammary epithelial cells (months 1-24)

a. Completed, 2002. The cDNA libraries were prepared in pBD prey vector with mRNA derived first from asynchronous MCF-7 cells and then from primary breast carcinoma samples. The former was constructed as a directional library while the latter was prepared as a non-directional library.

b. Completed, 2002. Both cDNA libraries were screened such that all mRNA in the cells is represented in the library screen. The MCF-7 library was superior to the primary breast library and was used for subsequent analyses.

c. Completed, 2002. The resultant colonies were picked, streaked and retested for specificity of interaction on selection medium +/- 5-FOA +/- methionine to induce prey expression. Master plates to retain yeast for future work were also prepared.
d. Completed, 2002. Those colonies that showed interaction with MycNTD in a prey fusion specific manner were processed further. To isolate prey, high-quality plasmid DNA was isolated by Qiagen or Clonetech prep and then transformed by electroporation into electro-competent DH5alpha cells.
e. Completed, 2003. Resultant mini-prep prey vector DNA were retested for functional interaction against the MycNTD using the RTA.
f. Completed, 2003. Those that repeated were tested for interaction with MycNTD containing deletion and point mutations in MbI and MbII.
g. Completed, 2003. Those that were dependent upon these highly conserved regions were subsequently sequenced.
h. Completed, 2003. These were analyzed for known and novel cDNAs and interaction further confirmed in vitro by GST fusions, in vivo by co-immunoprecipitation of ectopically expressed and tagged proteins and finally by in vivo co-immunoprecipitation of endogenous proteins.
i. Completed, 2004. We focused in on one Myc interactor, GSK-3 alpha, that was cloned using the RTA as described. Ectopic expression of the tagged protein was analyzed for interaction (Fig 2 and Fig 3) and by immunofluorescence to determine subcellular localization relative to Myc (Fig 4).
j. Completed, 2004. The Myc-binding protein, GSK-3 alpha, was tested for its ability to alter Myc function. Ectopic expression or RNAi knock-down of GSK-3 alpha had no effect on Myc autosuppression, apoptosis, growth, transformation. Pharmacological inhibitors to GSK-3 were also used to evaluate the effect of Myc's biological function, but no differences were evident.
k. Completed, 2004. The expression of Myc and the Myc-binding proteins was analyzed for expression and activity in cell lines and primary tissue samples derived from normal and transformed mammary carcinomas. GSK-3 is ubiquitously expressed.

Task 3. To identify functional inhibitors of Myc by targeting and blocking MycNTD interaction with key cellular binding proteins (months 18-36)

a *Altered and approved, 2003. Establish MycNTD and novel interactor fused to TUP1 in yeast strain designed for the inhibitor screen. In this system, interaction causes death of yeast upon selection. Peptide aptamer combinatorial libraries are introduced into yeast and rapidly screened by selection for rescue of yeast cell death.
b * Altered and approved, 2003. Peptide is isolated, and retested for functional inhibition of MycNTD and the Myc-binding protein.
c * Altered and approved, 2003. These experiments are difficult to describe without the molecules in hand. For example, if the Myc-binding protein is known to bind other cellular proteins then it will be determined whether the peptide binds in a similar or unique manner than the natural cellular blocking protein. By this approach the mechanism of inhibition of the peptide can be determined. Moreover, specificity of interaction can be evaluated first in the RTA system against other cellular molecules with similar structure or function, and then in mammalian cells

d * Altered and approved, 2003. Assays to measure the efficacy, specificity and sensitivity of peptide inhibition of Myc and the Myc-binding protein interaction as well as Myc function will be tested in mammalian cells.

*These changes were approved in the 2003 Annual Report: The RTA system can work well as a screening system to identify inhibitors of protein:protein interactions, as proposed. However, evaluating this system to screen for peptides that can block Myc:TRRAP interaction, with the minimal regions of these proteins that interact, has resulted in an assay with a poor signal to noise ratio. The high background precludes its utility as a screening tool for this pair of interactors. To address the goals of Task3, we have adopted an alternative approach as outlined below.

Task 3. To identify functional inhibitors of Myc by targeting and blocking MycNTD interaction with key cellular binding proteins (months 18-36)

a. Completed, 2004. To identify the contact points between TRRAP and Myc, we initiated structural studies to analyze the smaller TRRAP and Myc NTD fragments identified by RTA and in vitro expression/interaction assays (Task 1, Part e). This work employed various structural and protein biochemistry techniques including CD spectroscopy and gel filtration, which was conducted in collaboration with Dr. Cheryl Arrowsmith. The results show that the TRRAP:Myc interaction detected by RTA can also be detected by CD spectroscopy (Fig 5). Experiments to further resolve the exact amino acid contact points in the complex were unsuccessful despite heroic efforts to produce high-quality polypeptide for NMR analysis (see Task 1, Part e above).
b. Not Applicable. Because the solution structure of TRRAP:Myc complex was not solved, we could not use this model as a guide to generate non-functional mutants of TRRAP and Myc that are interaction defective, as proposed.
c. Not Applicable. The detectable binding of the TRRAP and Myc NTD fragments by NMR would have provided a useful system to screen for peptide inhibitors of this interaction. However, without the structure, a high-throughput screen could not be conducted, as proposed.

Key Research Accomplishments

•A region of TRRAP essential for interaction with Myc has been identified using the RTA and in vitro interaction analyses (Fig 1 & 5).

•Two cDNA libraries have been constructed and screened to identify polypeptides that interact with the Myc N-terminus. Positive clones from the MCF-7 library have been isolated, re-screened and sequenced (Appendix A).

•Further analysis of Myc:GSK-3 interaction was conducted (Fig 2-4)

Reportable Outcomes

- Abstract presented at Reasons for Hope, Breast Cancer Conference, Orlando 2002
- Ph.D. degree conferred based on project supported by this award
- Employment opportunities received based on training supported by this award
- Two manuscripts now in preparation will be credited to DOD funding.

Conclusions

As described in the first specific aim, the RTA was employed to identify a region of TRRAP which is required for binding to the Myc N-terminal domain. As outlined, partial fragments of TRRAP and Myc were tested by the RTA as well as in vitro interaction analyses to identify the minimal regions within each protein that are critical for interaction. We had hoped to map the precise points of TRRAP/Myc interaction using Nuclear Magnetic Resonance (NMR), but despite several attempts using a variety of strategies, soluble polypeptide at the levels required to conduct NMR were not achievable. While we have exploited the TRRAP:Myc interaction in this pilot project, we expect to extend this strategy to the other Myc interactions isolated in Task 2. The lead compounds generated by this work have the potential to be further developed into novel therapeutics that block Myc function as an oncogene. A manuscript describing these results is presently in preparation.

As outlined in the second specific aim, two cDNA libraries were constructed and screened using the RTA to identify novel Myc binding proteins. Positive clones resulting from the MCF-7 library screen were further characterized to ensure they were true Myc interactors and their identity was revealed through nucleotide sequencing. Further analysis of GSK-3/Myc was conducted and a manuscript describing the screen (Appendix A) as well as the further analysis of GSK-3/Myc is presently in preparation. With these interactors in hand the molecular mechanism of action of Myc in the carcinogenic process will be further revealed. In addition, these clones will provide the essential experimental tools to develop additional therapeutics that will break Myc interactions that hold a key role in the transformation.

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2. McMahon, S. B., Van Buskirk, H. A., Dugan, K. A., Copeland, T. D., and Cole, M. D. (1998) The novel ATM-related protein TRRAP is an essential cofactor for the c- Myc and E2F oncoproteins. *Cell* 94, 363-74

3. Oster, S. K., Ho, C. S., Soucie, E. L., and Penn, L. Z. (2002) The myc oncogene: MarvelouslY Complex. *Adv Cancer Res* 84, 81-154

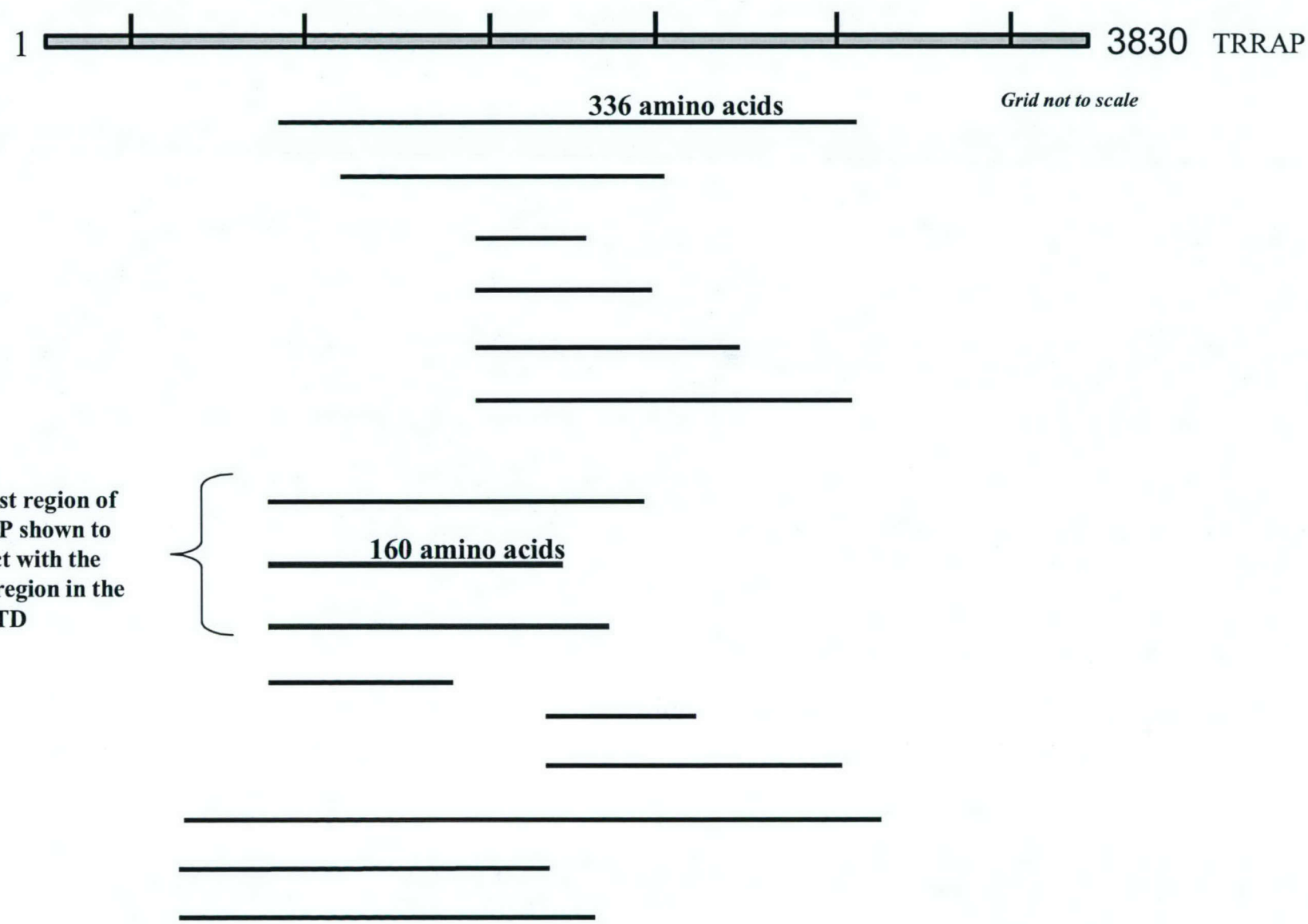
List of personnel receiving pay from this research effort:

Cynthia Ho
John Watson
Romi Ponzielli
Sam Kim

Appendices: Appendix A

Fig. 1

TRRAP fragments constructed, expressed and evaluated for interaction with the MBII region of the MycNTD



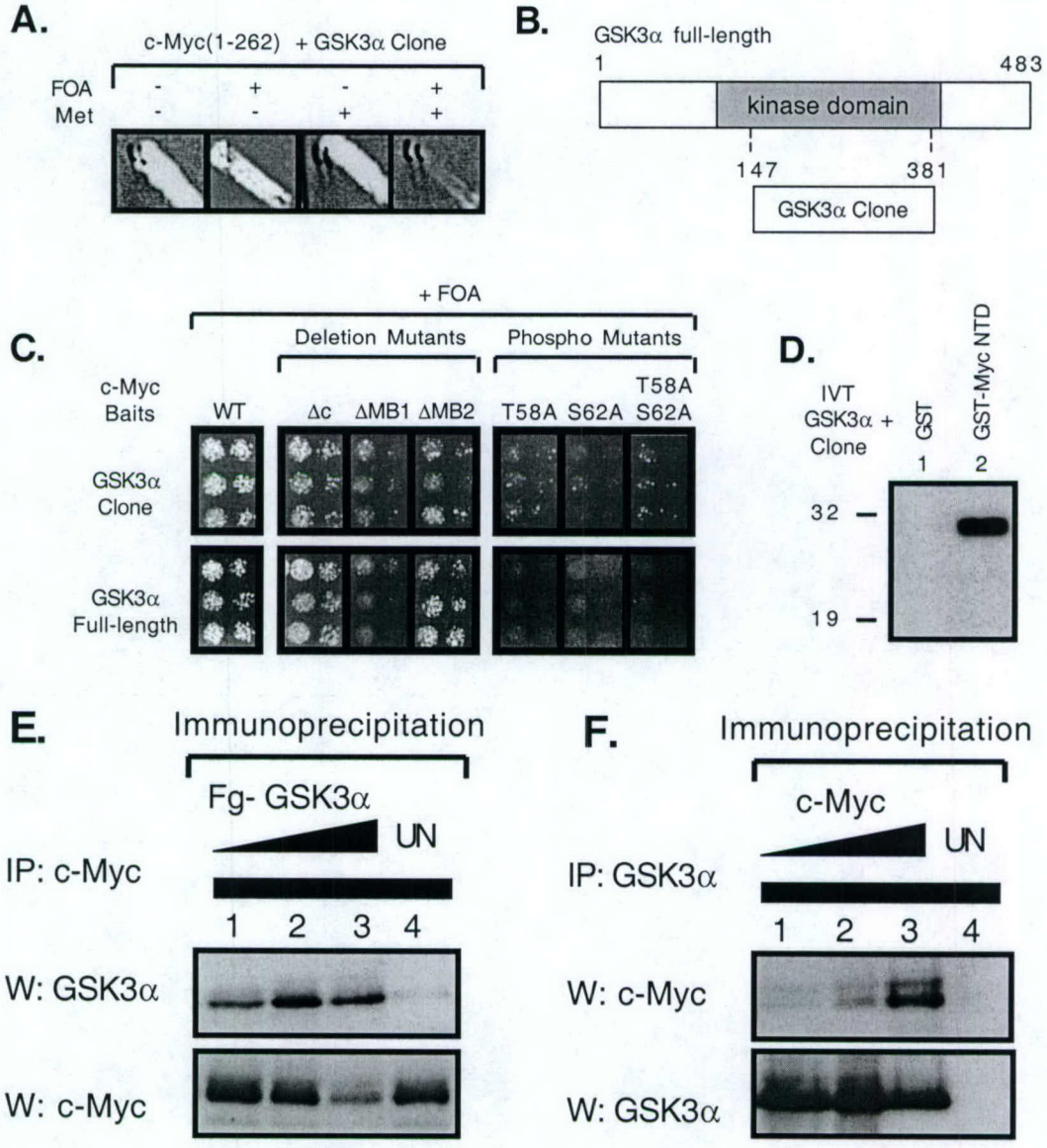
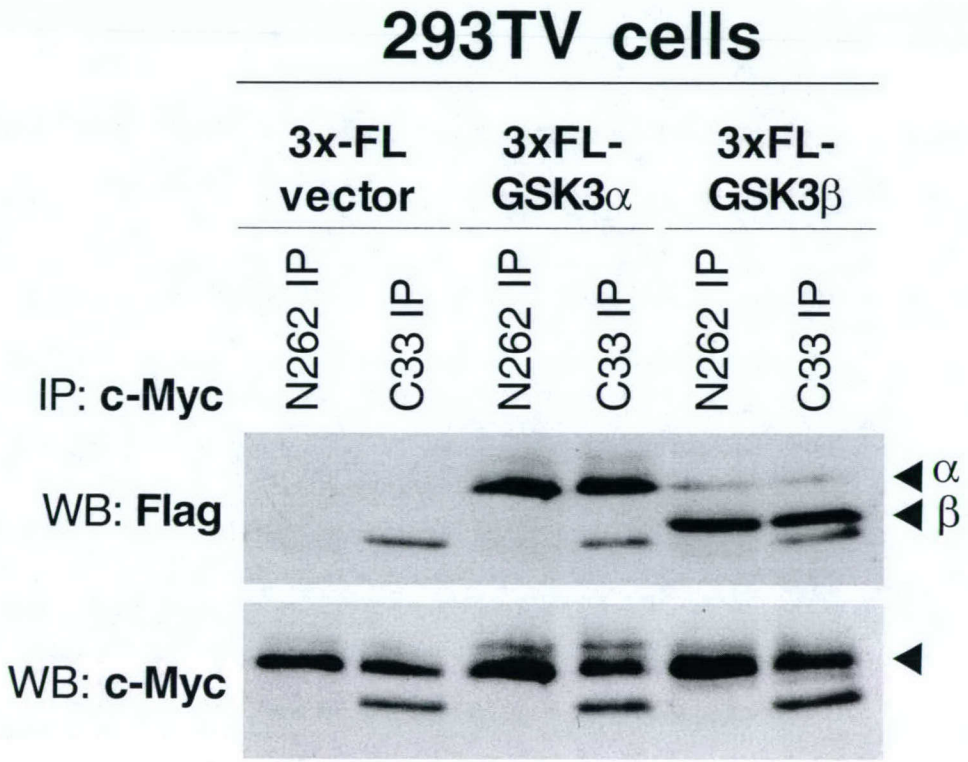


Figure 2: **(A)** RTA library screen identifying GSK3α kinase domain clone. **(B)** Schematic representation the partial GSK3α clone identified by RTA **(C)** Interaction of the GSK3α clone and full-length GSK3α with various Myc NTD baits. **(D)** Interaction of the translated GSK3α clone with GST-Myc NTD in vitro. **(E)** Immunoprecipitation of Fg-GSK3α full-length with c-Myc from mixed nuclear extracts. **(F)** Immunoprecipitation of c-Myc with Fg-GSK3α from mixed nucelar extracts.

Figure 3: GSK3α/β interacts with c-Myc *in vivo*



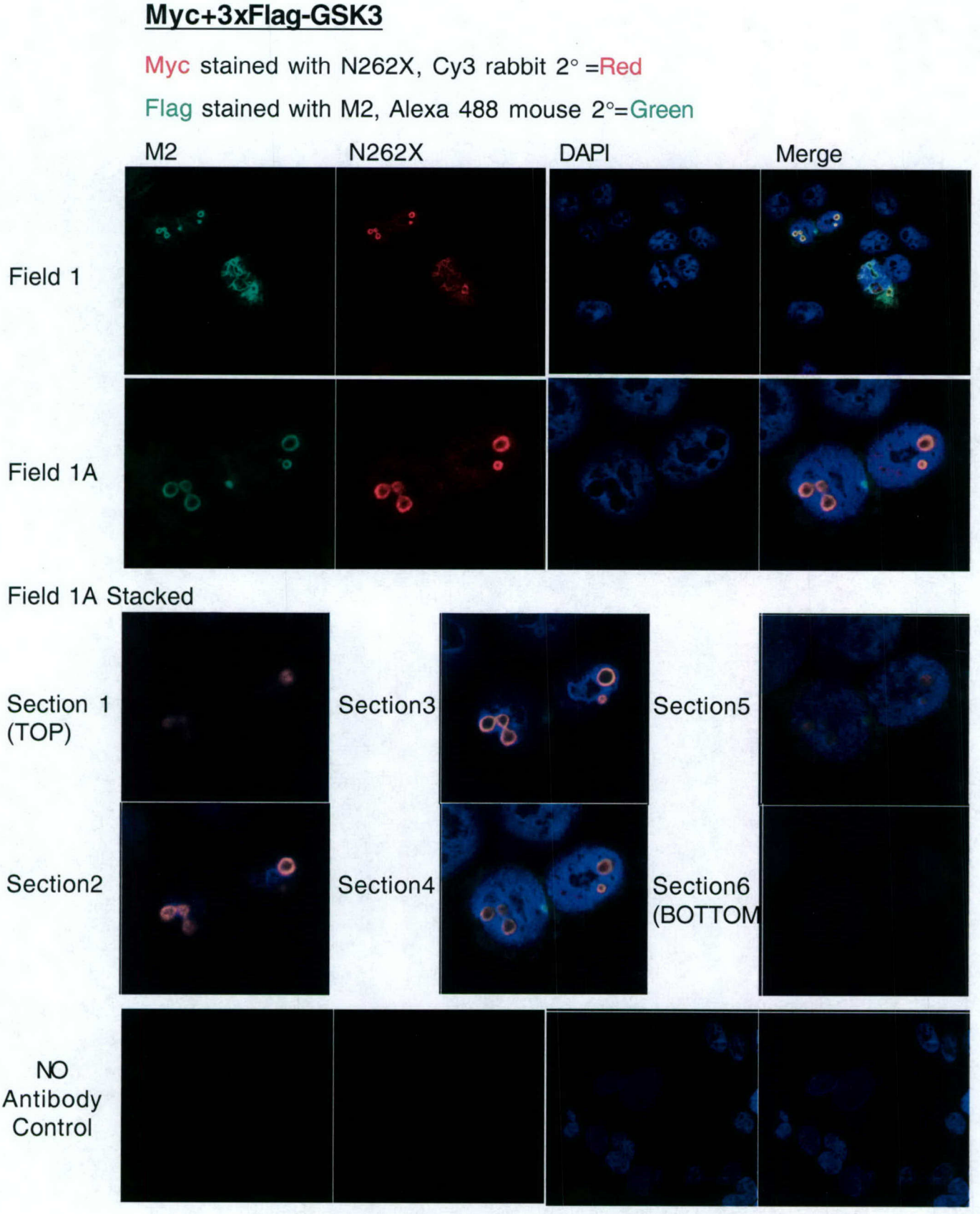
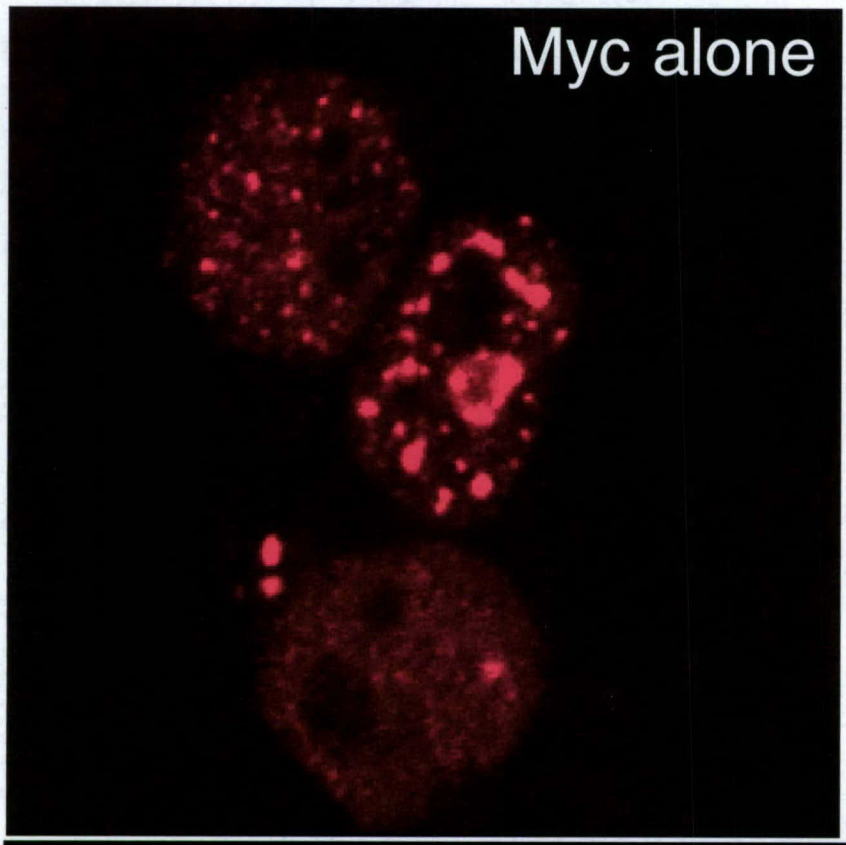


Figure 4a: Myc and ectopic GSK3 co-localize in unusual nuclear bodies.

Cy3 (Red) channel only



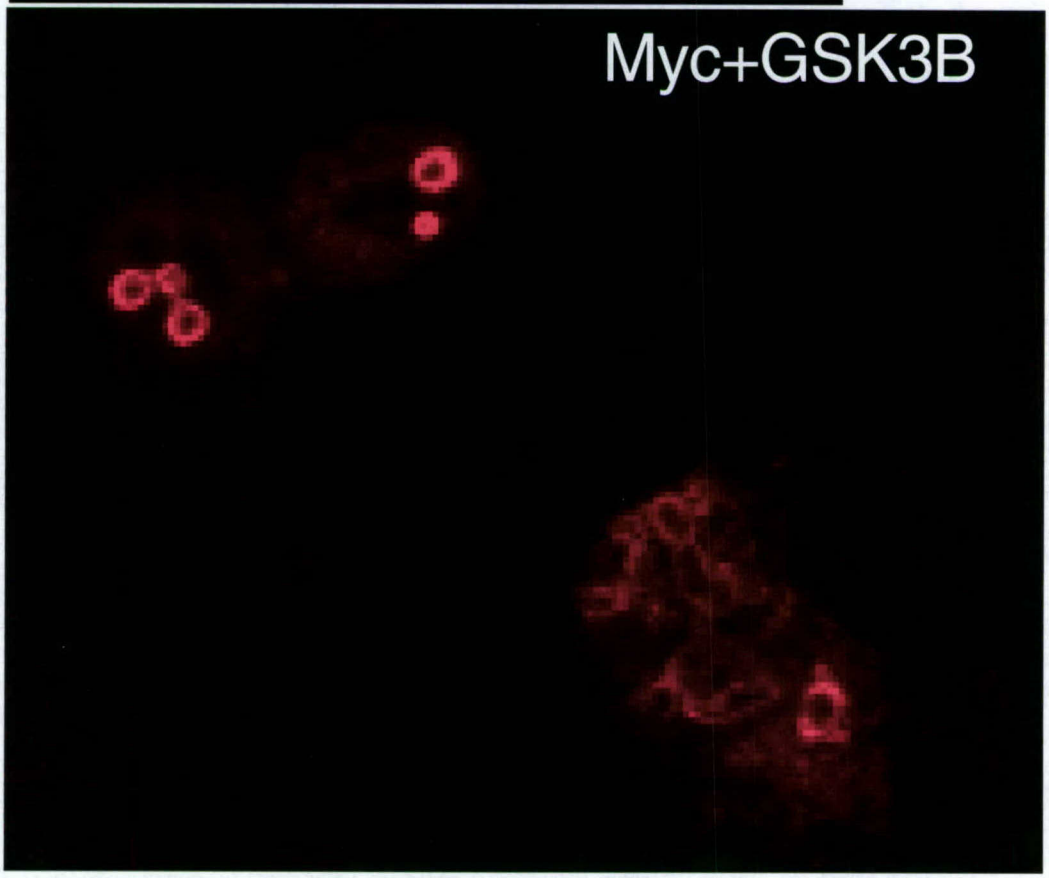


Figure 4b: Myc localization is altered when coexpressed with GSK3

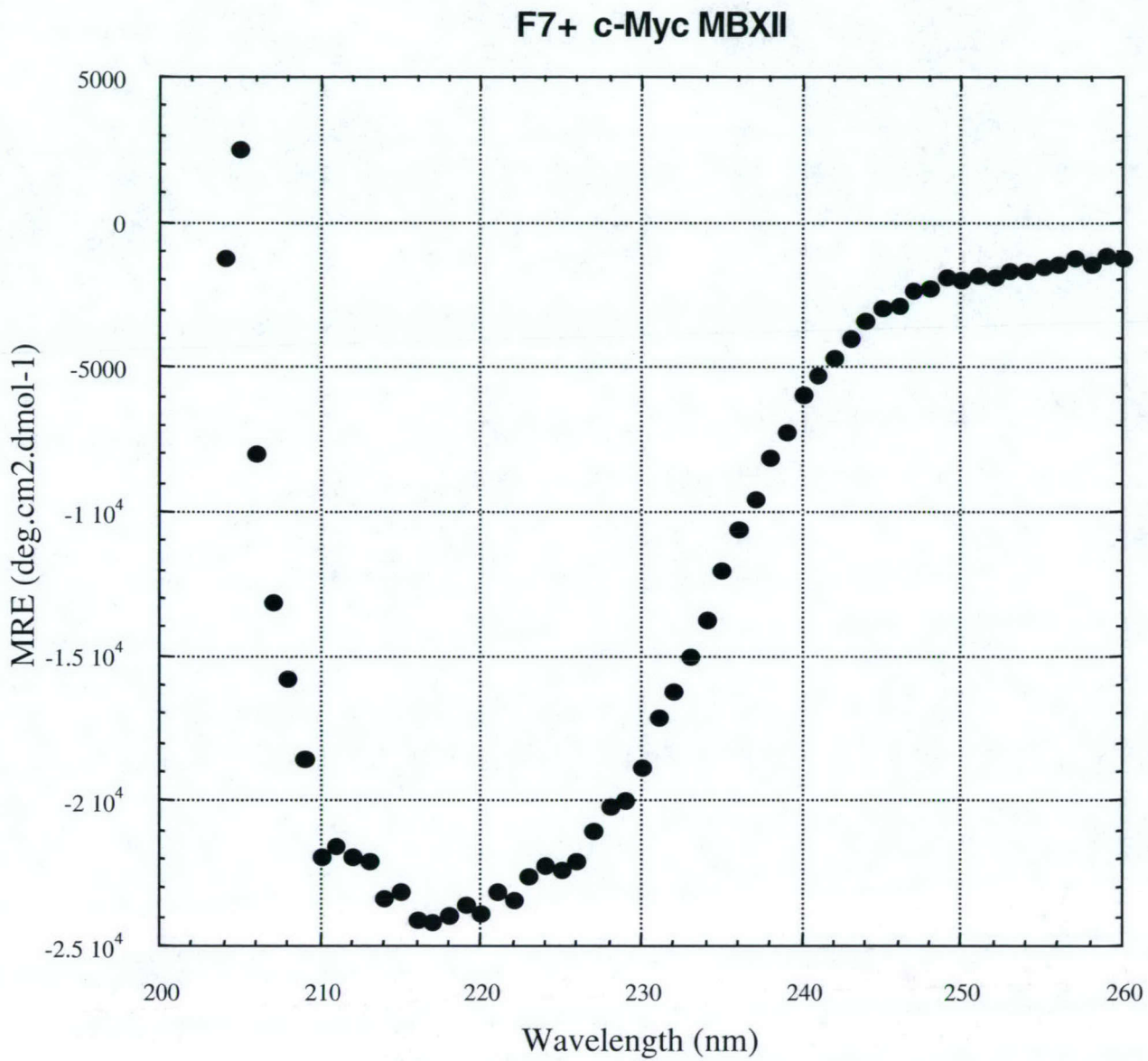


Figure 5: Circular Dichroism further suggests the 160 amino acids of TRRAP identified as the Myc-binding Region using the RTA and in vitro binding assays, interacts with the MBII region of the MycNTD

APPENDIX A

Identification of Myc N-terminal Domain Protein Partners Using the Novel Repressed Transactivator Assay

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Keywords
c-myc oncogene, transformation, novel yeast two-hybrid, protein interaction

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Abstract

The *c-myc* oncogene encodes a 439 amino acid protein that plays a critical role in cell cycle entry, apoptosis, proliferation, differentiation and, when deregulated, Myc contributes to a large number and wide variety of human cancers. Indeed, Myc's potent transforming potential and pervasive role in human malignancies has inspired intensive research into the molecular mechanisms behind its activity and regulation. Two highly conserved N-terminal domain (NTD) regions are necessary for all cellular activities of Myc and recent studies suggest their role is to recruit cellular cofactors necessary for Myc regulation and function. Currently, the identification, physical mapping and functional characterization of Myc NTD interactions has relied heavily upon labour intensive biochemical strategies. However, due to the expanding interest and increased urgency in the field to dissect Myc NTD function, a simpler and more efficient method to examine Myc NTD interactions is highly desirable. To this end, we have established a high-throughput screen termed the Repressed Transactivator Assay (RTA) to identify Myc NTD-specific binding proteins. We demonstrate that the RTA system can be used to such interactions in a sensitive and specific manner. We also performed a cDNA library screen and show that Myc NTD-binding events captured by RTA were also detected *in vitro*. Importantly, the RTA identified an interaction with the kinase domain of GSK3α, which has been implicated in phosphorylation of the Myc N-terminus *in vivo*. The initial leads generated by the RTA will provide a cohort of Myc NTD-specific interactors by which to study the motifs and structural point of interaction responsible for Myc regulation and function using conventional biochemistry and structural biology techniques.

Introduction

Deregulation of the *c-myc* oncogene is found in a large number and wide variety of human cancers. The activation of *c-myc* often leads to increased Myc protein levels, which has been correlated to poor prognosis and a more aggressive disease phenotype. In normal cells, Myc expression is highly regulated and its levels are rapidly induced by mitogenic stimulation, which is a pre-requisite for cell cycle entry. Thus, when deregulated, the strong growth signal imposed by Myc is a potent promoter of tumorigenesis.

The Myc protein is a nuclear phosphoprotein of 439 amino acids that can be divided into two main functional regions. At the C-terminal domain (CTD), a basic helix-loop-helix leucine-zipper (bHLHLZ) motif is responsible for site-specific DNA-binding and heterodimerization of Myc with its cognate partner Max. The N-terminal domain (NTD) encodes for sequences with activation potential, which can be divided into glutamine (Q), proline (P) and acidic-rich regions (Figure 1A). Also found within the NTD are two critical domains, termed Myc Box 1 (MB1) and MB2. These highly conserved sequences are important to all known functions of Myc, including transformation, proliferation, apoptosis and block to differentiation (reviewed in Oster et al, 2002). However, the exact function of these critical sequences in the regulation of Myc biology remains unclear. Recent studies have brought new insights and it is now evident that these regions are important for the regulation of Myc target genes, the selective expressions of which are thought to be responsible for Myc activity. Moreover, an essential cofactor in Myc-mediated activation and transformation, TRRAP

(Transformation/transactivation domain-associated protein), was recently identified and found to interact with Myc at these conserved sites (McMahon *et al.*, 1998; McMahon *et al.*, 2000). Thus, one critical function of the Myc NTD may be to recruit essential cellular factors required for Myc activity.

To gain a better understanding of Myc protein function at the molecular level, we sought to establish a high throughput screen to identify novel cofactors bound to Myc at the N-terminus. Currently, the identification, physical mapping and functional characterization of Myc NTD interactions has relied heavily upon labour intensive biochemical strategies. However, with the expanding interest and increased sense of urgency in the Myc field to understand Myc NTD function, a simpler and more high throughput method to examine Myc NTD interactions would be highly desirable. In the past, the investigation of Myc NTD-binding proteins has not generally involved yeast two-hybrid strategies due to the strong activation conferred by the Myc NTD when used as bait in these systems (Fields and Song, 1989). In recent years, alternative two-hybrid approaches have become commercially available that can accommodate the use of self-activating baits by moving the assay out of the nucleus and switching to reporter systems that do not use transcriptional activation as the method for interaction detection (Aronheim *et al.*, 1997; Johnsson and Varshavsky, 1994). Because Myc normally functions as a nuclear transcription factor, we reasoned that it would be most advantageous to allow the Myc NTD to function in its normal cellular setting and to employ a reporter system that would take advantage of Myc's inherent ability to activate. To this end, a novel yeast two-hybrid screen termed the Repressed Transactivator Assay

(RTA) was developed and we have now established its use with the Myc NTD as bait (Hirst et al, 2000).

We demonstrate that the novel RTA system can be used to identify interactions at the Myc NTD in a sensitive and specific manner. We also performed a cDNA library screen using the Myc NTD as bait and show that binding events captured by RTA were also detected *in vitro*.

Materials and Methods

RTA constructs and Yeast strain:

The bait cDNAs were cloned into the pG yeast expression plasmid as fusions to the Gal4 DNA-binding domain (DBD, aa 1-147). Gal4-Myc NTD was constructed using sequence encompassing amino acids (aa) 1-262 of wildtype human c-Myc, which was excised from GM(1-262) (Ref) using XhoI/SmaI restriction digest, blunted and cloned into pG at SmaI. Gal4-ΔMB1 (deleted MB1, aa 43-65), Gal4-ΔMB2 (deleted MB2, aa 128-145) and Gal4-W135E Myc mutant constructs were constructed by PCR amplification of mutated human templates previously described (Oster et al, 2002). Gal4-N-Myc and Gal4-L-Myc expressing vectors were cloned by PCR amplification of the region directly homologous to c-Myc 1-262 of each respective wildtype human template. N-Myc 1-XXX was cloned using X/Y and L-Myc 1-XXX was cloned using X/Y. Full-length adenovirus E1A oncoprotein was amplifed by PCR from 12S template with primers encoding X/Y ends. The activation domains of herpes simplex VP16 protein (aa)

and heat shock transcription factor 1 (HSF1, aa) were also cloned by PCR using X/Y and X/Y ends respectively.

The control TUP1-fusion proteins were cloned into the yeast expression vector pBDH as fusions to the TUP1 repression domain (RD, aa 1-200). Full-length human α-tubulin was cloned using X/Y digestion and ligation into pBDH. Mutagenesis to remove a TGA stop codon in the fusion region of TUP1-α-tubulin was performed using Quikchange mutagenesis kit (Stratagene). Full length human TBP was PCR amplified with EcoRI and BamHI restriction site ends for cloning. Full length pRB-family inserts were used and cloned as follows: p107 was excised and cloned using X/Y, pRB excised and cloned using X/Y, p130 excised and cloned using X/Y from XXX. Full-length human GSK3α was excised from XXX and cloned into pBDH at EcoRI. All fusion regions and open reading frames were sequence verified. PCR primer sequences used in the cloning of these constructs are be available upon request.

The Mav108 reporter yeast strain has been previously described (Vidal *et al.*, 1996).

Solid and liquid yeast growth media:

Wildtype Mav108 yeast cells were grown in complete yeast growth media in preparation for transformation. Single transformants were grown on –tryptophan (-trp) plates for propagation of Gal4-fusion bait vectors alone or –histadine (-his) plates for propagation of TUP1-fusion prey vectors alone. Cotransformants were grown on –tryptophan-histadine-methionine (-trp-his-met) triple dropout plates. Where required L-methionine was added to the –trp-his-met dropout media to a final concentration of 2mM

(Sigma M-). 5-flouroorotic acid (FOA) was added directly to the media after sterilization to a final concentration of 0.05% (Toronto Research Chemical, F-). Liquid media was made in the same manner as solid plating media except that agar was omitted.

Lithium acetate (LiOAc) Yeast transformation:

Freshly prepared wildtype Mav108 cells were used in all transformation reactions. Yeast cells in log-phase growth were harvested by centrifugation and aliquoted into lithium acetate mediated transformation reactions containing the appropriate combination of bait and prey plasmids. Reactions were incubated for 30 minutes prior to a 15 minute heat shocked at 42°C with an addition of 10% DMSO just prior to heat shock. Each reaction was then plated onto the appropriate drop-out selection and resulting colonies incubated for 2-3 days at 30°C.

FOA plating and Liquid β-galactosidase assays:

FOA selection platings were performed with freshly transformed Mav108 cells. Transformed yeast cells were picked, suspended in sterile ddH_20 and diluted to 1/10 and 1/100 with additional sterile ddH_20. All dilutions were performed in triplicate. A sample of each dilution (undiluted, 1/10 and 1/100) was then transferred by spotting onto +/- FOA plates and incubated at 30°C. Results were documented using Kodak gel doc system.

Liquid β-galactosidase assays were also performed using freshly transformed cells that were grown overnight in liquid culture. Cells were pelleted, lysed and subject to

standard analysis of β-galactosidase activity using ONPG substrate. All samples were tested in triplicate.

Immunoblot analysis:

Yeast protein lysates were made using SDS/urea buffer and electrophoretically separated on a 12% polyacrylamide gel. Specific bands corresponding to the TUP1-fusion proteins were detected using a monoclonal αTUP1 antibody at 1:1000 concentration in 5% skim milk + 0.1% Tween20 solution. Bands were visualized using standard chemiluminscence reagents according to manufacturers' protocol (Pierce).

MCF-7 cDNA Library Synthesis and Screening:

MCF-7 cDNA library synthesis has been described elsewhere (Hirst et al, 2000). A survey of 30 random library clones revealed a ligation efficiency of greater than 90% with an average cDNA insert size of 1.5kb. A total number of 1.6×10^6 primary library transformants was obtained.

The Gal4-Myc NTD bait and MCF-7 cDNA library vectors were serially transformed into Mav108 cells. Transformation reactions were plated directly onto –trp-his-met +FOA screening plates. A total of XXX cDNAs were screened. Surviving colonies were transferred to –trp-his-met master plates for passage and storage. Master plate clones were then tested on +/-FOA,+/-Met selection plates for specificity of growth. Subsequent screening by replating and activator bait panel tests was performed using extracted library plasmids that were retransformed into fresh Mav108 cells.

Extraction of Library plasmid and sequence analysis:

YPER reagent (Pierce). Electroporation into homemade cells.

In vitro expression, binding and competition experiments:

Library cDNA inserts were PCR amplified to include a T7 promoter sequence (double underline), a kozak consensus (bold) and 3xATG methionine codons, sequence specific to pBDH is underlined, primer 1: 5' CC ctc gag TAA TAC GAC TCA CTA TAG GGa g**CC ACC ATG** ATG ATG CAG CAA CCA CCT CCC CAG GTT TCC GTG GCA G 3'; primer 2: 5' ggc atg ccG ACC AAA CCT CTG GCG AAG AAG TCC AAA GCT CGG G 3'. ^{35}S-methionine labeled library proteins were produced from the PCR templates using an in vitro reticulocyte lysate system (TNT T7 Quick for PCR DNA, Promega and Redivue L-[^{35}S]methionine in vivo cell labeling grade, Amersham). Myc NTD wildtype and mutant templates were cloned into pGEX-2T GST-fusion expression vector and expressed recombinantly. Crude lysates of each were made in XXX buffer.

In vitro binding reactions were performed as follows. Labeled library products alone were first incubated in a preclear reaction with only the glutathione sepharose beads in XXX buffer. Reactions were pelleted and beads from preclear reaction were kept on ice to be processed later with the binding reactions. Supernatants from the preclear reactions were transferred to tubes containing GST-fusion protein and incubated at 4°C for 1 hour with constant agitation. Equal amounts of glutathione sepharose beads were then added to each binding reaction and tubes returned to 4°C for a further 1 hr

incubation. After centrifugation, collected bead pellets of binding and preclear reactions washed three times using 1 ml of XXX buffer per wash with a transfer to fresh tubes after the second wash. 2xSDS loading buffer was added and samples boiled to release bound proteins from beads. Bound proteins were electrophoretically separated on 12% polyacrylamide and bands detected by autoradiography. Competition experiments were performed using the same conditions as binding reactions except that purified GST-free Myc NTD was titrated into the competition reactions in the indicated amounts. Reactions proceeded and were processed as described above.

Results

Outline of the basic components of the Myc RTA assay and reporter gene system

Schematic outline of the basic components of the RTA system are detailed in Figure 1. Wildtype Myc sequence from amino acids 1-262 (Figure 1A, black bar), which includes the functionally important Myc Box regions, was cloned in-frame to the Gal4 DNA-binding domain (DBD) to be used as bait (Gal4-Myc NTD). In contrast to other two-hybrid strategies, the RTA requires strong activation of the reporter system by the Gal4-fusion bait as interactions are detected by the suppression, rather than activation, of reporter gene expression. This is achieved by fusing prey proteins to the repression domain (RD) of TUP1, a general yeast repressor protein, so that upon interaction of the Gal4-Myc NTD bait with a TUP1-fusion prey, expression from the reporter will be inhibited. We established the RTA using the Mav108 yeast cell strain, which contains both *ura3* and *lacZ* reporter genes (Vidal et al, 1996). Activation of the *ura3* reporter leads to the death of yeast cells when plated onto FOA-containing media (ref). As such,

expression of the activating Gal4-Myc NTD bait alone or in the presence of a non-interacting prey, the *ura3* reporter remains "ON" and the yeast cells fail to grow on +FOA. Similarly, activation of the *lacZ* reporter in the presence of Gal4-Myc NTD alone or in the absence of interaction, leads to the accumulation of cleaved o-nitrophenol galactoside (ONPG) substrate that is detected as a yellow color change in standard liquid β-galactosidase assays (Figure 1B). However, when the Gal4-Myc NTD is coexpressed with an interacting TUP1-fusion prey, the reporter genes are turned "OFF" allowing the cells to grow in +FOA media or resulting in quantitatively less cleaved ONPG substrate and a relatively clear liquid β-galactosidase reaction solution (Figure 1C).

Control interactions for Gal4-Myc NTD and Gal4-E1A baits were detected by RTA using both reporters

An important pre-requisite for the RTA system is the ability of the bait fusion to confer adequate reporter gene activation. Mav108 yeast cells were transformed with empty pG bait vector, Gal4-Myc NTD or Gal4-E1A alone. Plating onto +FOA clearly revealed that control cells transformed with pG, which has no activation potential, were able grow on +/-FOA conditions to a similar extent (Figure 2A). By contrast, yeast cells expressing either Gal4-Myc NTD or Gal4-E1A were unable to grow on +FOA due to *ura3* reporter gene expression. This lack of viability was not due to the transformation procedure or to a general toxicity of the expressed bait since both Gal4-Myc NTD and Gal4-E1A transformants were able to grow on control -FOA media. It was important to observe that cells transformed with the TUP1-fusion preys alone were able to grow to a similar extent on either + or -FOA conditions, indicating that the proteins were not toxic

to yeast cell growth nor could they activate *ura3* gene expression on their own (Figure 2B).

The Gal4-baits were then cotransformed with a control panel of TUP1-fusion prey proteins and when plated onto +FOA. Only those cells harbouring an interacting pair were able to grow (Figure 2C). Again, cells plated onto control FOA-free media were fully viable indicating that the observed lack of growth on +FOA was not due to a general lack of viability (data not shown). Positive control TUP1-fusion preys were cloned from full-length templates of known Myc NTD-interacting proteins, including α-tubulin (Alexandrova *et al.*, 1995), TATA-binding protein (TBP) (Hateboer *et al.*, 1993; Maheswaran *et al.*, 1994; McEwan *et al.*, 1996)and p107 (Beijersbergen *et al.*, 1994; Gu *et al.*, 1994), which conferred significant rescue of growth when coexpressed with the Gal4-Myc NTD bait (Figure 2C, upper panel). In contrast, non-interacting negative control Myc NTD TUP1-fusions, pRB, p130 and pBDH empty prey vector, were unable to rescue yeast cell growth on +FOA. When this panel of positive and negative Myc NTD interactors was then coexpressed with the Gal4-E1A bait, a different pattern of growth was detected on +FOA (Figure 2C, lower panel). Because E1A encodes many of the same biological activities of Myc and because it is has well-established interaction with TBP (Molloy *et al.*, 1999) and the pRB-family of proteins, it was used as a specificity control in the experiment. As expected, Gal4-E1A expressing cells were strongly rescued by coexpression of TBP and the pRB-family proteins, p107, pRB and p130. Under these experimental conditions no significant growth was detected for cotransformants of Gal4-E1A and either α-tubulin or the pBDH negative control suggesting that these proteins do not interact.

Quantitation of *lacZ* reporter gene activity by liquid β-galactosidase assay in Gal4-Myc NTD cotransformants revealed a pattern of *lacZ* reporter gene activity that supported the *ura3* reporter observations on +FOA and correlated with the presence of positive and negative TUP1-fusion prey interactions. All β-galactosidase assays were performed in triplicate and a representative data set is shown in Figure 2D. Positive control TUP1-fusion preys, α-tubulin, TBP and p107, had significantly lower relative β-gal units as compared to the pBDH vector control or to pRB and p130 negative controls, which was indicative of interaction. Importantly, concordance between the *ura3* and *lacZ* reporters showed that the RTA system was able to differentiate between positive and negative Myc NTD interactions using reporters that have distinct promoter configurations indicating that the positive interactions detected were not promoter-context dependent.

Although appropriate TUP1-fusion prey protein expression can be inferred from the results shown in Figures 2C and 2D, immunoblot analysis was performed to ensure that each prey could be detected and at the expected migration. Furthermore, this would clarify that the failure to detect an interaction was not due to lack of fusion protein expression. The TUP1-fusions were expressed, as detected by a αTUP1 monoclonal, and migrated to the correct predicted molecular size (Figure 2E, specific TUP1-fusion bands marked by an arrow). Some variability in the abundance of the different fusion proteins was observed, however, all non-interacting control TUP1-fusions, pRB, p130 and pBDH were clearly detected excluding the possibility that negative results generated by these preys was due to lack of protein expression.

Rescue of growth on +FOA is dependent on the concurrent expression of the interacting TUP1-fusion

To determine if the growth rescue by the positive controls would still be maintained on +FOA in the absence of an interacting prey, TUP1-fusion protein expression was selectively eliminated. TUP1-fusion prey expression is controlled from a regulatable yeast Met3p promoter that can be repressed in the presence methionine (+Met), allowing for the inhibition of prey protein production by a simple change in growth conditions. Mav108 yeast cells were cotransformed as before with the Gal4-Myc NTD and control TUP1-fusion preys, except that each reaction was equally divided and plated onto +Met and -Met growth conditions. Colonies of equal number and size were observed between the + and -Met growth conditions indicating that the high methionine environment had no detrimental effect on either transformation efficiency or yeast cell growth (data not shown).

The resulting –Met and +Met cotransformants were plated in parallel and concurrently onto all four selection conditions denoted -FOA, +FOA, -FOA+Met and +FOA+Met. If the observed growth on +FOA is specific to the concurrent expression of an interacting prey then plating onto +FOA+Met conditions would lead to the loss of rescue when expression of the interacting TUP1-fusion is suppressed. A representative experiment is shown in Figure 3A. Both –Met and +Met cotransformants were able to grow on control FOA-free media in the presence or absence of additional methionine, indicating that these cells were fully viable (Figure 3A, see -FOA and –FOA+Met panels). Plating onto +FOA media showed that both TBP and p107 TUP1-fusion preys were efficient at rescuing yeast cell growth of either –Met or +Met grown

cotransformants. Importantly, comparable levels of +FOA rescue were achieved from both –Met and +Met grown colonies, which indicated that the suppression of prey expression under +Met was efficiently reversed when the cells were shifted back to methionine-free growth conditions. More importantly, when the cells were plated onto +FOA+Met media, no significant rescue of yeast cell growth could be detected. This indicated that the observed growth on the matching +FOA plate was indeed dependent on the expression of the interacting TUP1-fusion prey (Figure 3A, see +FOA+Met panels).

Loss of TUP1-fusion protein expression under +Met growth condition was confirmed by αTUP1 immunoblot analysis. The different TUP1-fusion proteins could be readily detected in cotransformants grown in -Met liquid media (Figure 3C, see -Met media panel). By comparison, prey proteins were undetected by αTUP1 analysis in +Met grown cells indicating a strong suppression of TUP1-fusion expression, which supports, at the protein level, those results observed on +FOA selection (Figure 3B, see +Met media panel).

Differential binding of TUP1-fusion positive control preys to mutant Myc NTD baits

Next we constructed a panel of Myc NTD mutants to test the Myc RTA system for the ability to detect differences in interaction among mutant baits with the control preys. The mutant Myc NTD panel included deletion mutants of conserved MB1 and MB2 regions, designated ΔMB1 and ΔMB2 respectively, and a point mutation in MB2 resulting in an amino acid substitution of a residue shown to be critical for Myc NTD activity and interaction (W135E) (refs).

Each Gal4-Myc NTD mutant bait was first tested to ensure that an adequate level of reporter gene activation would be achieved. Deletion of MB1 and MB2 or substitution at W135E did not result in detectable defects in *ura3* activation and the Myc mutants were as efficient as wildtype at killing the cells on +FOA (Figure 4A).

Coexpression of the wildtype Gal4-Myc NTD with either TBP or p107 TUP1-fusion preys again resulted in significant growth on +FOA when compared to the negative pBDH empty vector control (Figure 4B). The mutant Myc NTD baits were then tested for interaction with TBP and no significant changes in the level of growth rescue were observed on +FOA for ΔMB1, ΔMB2 or W135E. Liquid β-galactosidase analysis of the deletion mutant baits with TBP showed that both mutant baits produced similar relative β-gal units as compared to wildtype Gal4-Myc NTD, which were as a group significantly different from the pBDH vector control (Figure 4C). This suggested that loss of either MB1 or MB2 did not significantly alter interaction of the Myc NTD with TBP. However, when the mutant Myc baits were coexpressed with p107, a qualitative decrease in yeast cell growth was reproducibly observed for ΔMB1 cotransformed cells on +FOA selection. This loss of yeast cell viability on +FOA with p107 was not observed to any significant extent with either ΔMB2 or W135E mutant baits. Liquid β-galactosidase experiments supported those results on +FOA and revealed that cells cotransformed with Gal4-ΔMB1 and p107 did have a significant elevation of β-gal units above that of the ΔMB2 mutant bait or the wildtype Gal4-Myc NTD control (Figure 4C). Further experiments are required to fully understand the significance of this observation, however, this data suggests that MB1 may be an important region of interaction between the Myc NTD and p107.

Identification of novel Myc NTD interacting proteins by library screen using RTA

To identify novel Myc NTD-interacting proteins, the Myc RTA system was used to screen a human cDNA library, which was derived from a MCF-7 transformed breast carcinoma cell line and constructed specifically for use in the RTA. The Gal4-Myc NTD bait and MCF-7 TUP1-fusion library were serially introduced into the Mav108 yeast cells and approximately $1.5x10^6$ transformants were plated directly onto +FOA screening plates. Library clones that were able to grow on +FOA during the screening period were transferred to FOA-free master plates for continued passage and storage. The library clones were then assessed for their ability to again grow on +FOA and to determine whether this growth was dependent on the concurrent coexpression of the library fusion by observing their phenotype on +Met media. This involved transferring of library clones from the master plate to a series of selection plates containing +/-FOA and +/-Met media conditions. A representative set of selection plates are shown in Figure 5A.

The response of library clones to the various media conditions was closely monitored and four pattern of growth were clearly distinguished (Figure 5B). Group 1 clones were viable when picked from screening plates onto master plates, but did not grow when transferred onto selection plates. Group 2 clones were able to grow on selection plates, but did not confer any significant rescue of growth when plated onto +FOA media. Library clones from groups 1 and 2 were not carried any further. Group 3 clones displayed impressive growth on +FOA, but could also grow on +FOA+Met media, which suggested that the growth rescue could be non-specific. Group 4 clones exhibited the ideal pattern of selection showing strong growth rescue on +FOA and specific loss of

this rescue when transferred to +FOA+Met media. These clones were brought forward into the next screen.

Retransformation test of selected library clones and TUP1-fusion protein analysis

Library vectors from group 4 clones were extracted and purified. In conjunction with sequence data, those clones determined to translate in-frame fusion proteins were tested. The data for a panel of select library clones, which represents the spectrum of results generated, is shown.

To determine if the expression of the library preys was toxic to yeast growth, library vectors alone were transformed into yeast cells. The transformants were able to grow on –FOA media and showed no significant change in viability when plated onto +FOA selection (Figure 6A). To eliminate false positives arising from reporter mutations, all library vectors were retransformed with Gal4-Myc NTD and tested again on +/-FOA and +/-Met selection plates for reconstitution of their original interaction phenotype in fresh yeast cells. A small number of clones did not repeat their original rescue phenotype on +FOA and were discarded (data not shown). Most library proteins were able to rescue growth on +FOA after retransformation and a representative selection of data is shown in Figure 6B. As seen previously, all cells were able to grow to a similar extent on control FOA-free media +/-Met. However, a distinct loss of yeast growth was observed on +FOA+Met media, which indicated specificity of growth rescue to the concurrent expression of the library protein (Figure 6B).

Immunoblot analysis under +/-Met conditions established that efficient inhibition of library prey expression had indeed occurred (Figure 6C, compare between – and +

lanes, specific bands are marked by an arrow). More importantly, proper expression of the TUP1-fusion library proteins was confirmed by detection of a specific band at the expected migration.

Activator panel test of library clones for interaction with Myc-specific and non-specific baits

To further examine the specificity of the library proteins for interaction with the Gal4-Myc NTD prey, those clones harbouring in-frame TUP1-fusions were then coexpressed with a panel of Myc NTD-related and unrelated activator baits. Related Gal4-activator baits included c-Myc-family proteins, N-Myc and L-Myc, which were constructed using wildtype sequence that was directly homologous to the amino acids 1-262 of c-Myc. Mutant Myc NTD baits included those previously used in Figure 4. Unrelated activator baits included full length E1A, used previously as a specificity control (Figure 2), and the activation domains of VP16 and HSF1.

This experiment was successful in identifying the library clones with differential ability to interact with the various activator baits. For comparison, TBP and pBDH expressing control cotransformants were also tested. As seen previously, TBP was able to confer robust growth when coexpressed with c-Myc NTD wildtype or mutant proteins, as well as with full length E1A (Figure 7). Importantly, no growth was detected for any of the pBDH cotransformants indicating that the TUP1 portion of the fusion did not contribute to interaction (Figure 7, bottom panel).

When the library proteins were tested, clones such as 2-20-1 and 6-11-1 appeared to confer a similar level of growth rescue across all baits suggesting non-specific

association with activation domains. Although clone 6-11-1 did not confer rescue of the W135E mutant bait, suggesting disruption to the wildtype Myc NTD sequence had an effect on interaction. In general, the library clones appeared to achieve the strongest growth when coexpressed with the Myc NTD and relatively little growth with the other related or unrelated activator baits (Figure 7, see clones 2-9-1, 11-26-1 and 9-11-1). This suggested that the observed response was specific to the c-Myc NTD. Cotransformation with the Myc NTD mutant baits gave rise to the most intriguing data, revealing that specific mutations could abrogate growth rescue by particular library preys and indicating regions of the Myc NTD that were potentially important for these interactions. A number of library preys had growth profiles similar to clone 9-11-1 where no significant grow was observed for any of the related, unrelated or mutant Myc NTD baits, suggesting that interaction was dependent on the wildtype Myc sequence.

In vitro expression, binding and specificity of wildtype GST-Myc NTD and mutant GST-ΔMB1 and GST-ΔMB2 fusions for ^{35}S-methionine labeled library proteins

To assess the ability of these proteins to bind the Myc NTD *in vitro*, each library clone was translated by a reticulocyte lysate system using radiolabeled ^{35}S-methionine from a T7 promoter-tagged PCR template of the cloned cDNA. A sample of each reaction was electrophoretically separated and translated products were detected by autoradiography. The most abundant product of each translation reaction migrated to the appropriate size corresponding to the specific library template released by SalI restriction enzyme digestion (Figures 8A, 1kb size marker indicated by an arrow and Figure 8B, specific translation products marked by an arrow). TBP and full length Max were also

translated, to serve as positive and negative controls for Myc NTD binding, respectively. Myc NTD wildtype and deletion mutant proteins were cloned in-frame with GST and expressed recombinantly. Crude lysates for each GST-fusion and GST alone were prepared and a sample of each was electrophoretically separated to determine their relative abundance. The GST proteins were the most abundant species of each respective sample and were expressed to approximately equivalent levels (Figure 8C, GST-fusion protein band marked by an arrow). The binding reactions were performed using unpurified crude lysates of the GST proteins since it was reasoned that the increase in reaction complexity conferred by the presence endogenous bacterial proteins would ameliorate background-binding events and enhance the specificity of the *in vitro* conditions.

To ensure that ^{35}S-labeled protein products were not binding to the glutathionine sepharose beads on their own, a preclear reaction for each sample was performed. This involved incubating the labeled library products with the glutathione sepharose beads, in the absence of GST-fusion protein, under the same conditions and concentrations as those used for the actual binding reaction. No significant activity was detected in precleared samples indicating that the labeled library proteins were not binding with the glutathione sepharose beads on their own (Figure 8D, lane 2).

To determine the specificity of library proteins for the Myc NTD in vitro, we first examined their ability to bind GST-Myc NTD wildtype versus their ability to bind control GST alone. Specific binding of GST-Myc NTD with the TBP positive control could be readily observed with an undetectable level of background in the GST alone lane (Figure 8C, compare between lane 4 and 4). As expected, the Max negative control

was not precipitated by either GST-Myc NTD or GST alone. Results for TBP and Max reactions indicated that the conditions of the binding experiment could correctly distinguish between the positive and negative controls *in vitro*. Similar to the TBP positive control, nearly all library proteins revealed a strong signal when incubated with GST-Myc NTD and no detectable signal when incubated with GST alone (Figure 8C, compare between lanes 3 and 4). For some proteins, a specific band could not be detected for either lane even after extended autoradiography (Figure 8D, see clone 2-20-1), suggesting that certain library proteins were unable bind with the GST-Myc NTD *in vitro*.

To further test the specificity of those library proteins that bound GST-Myc NTD, competition experiments were performed. For competition conditions, binding reactions were assembled as before except that an increasing amount of purified Myc NTD was titrated into the reaction, as indicated, to compete with GST-Myc NTD for the labeled library protein. Three concentrations of Myc NTD were tested and the results are shown in increasing order from left to right as 1/10 molar, equimolar and 4x molar concentrations of purified Myc NTD to GST-Myc NTD (Figure 8E). Binding of clone 9-11-1 was almost entirely competed by an equal concentration of Myc NTD to GST-Myc NTD. Clone 11-26-1 gave an intermediate result and showed a marked decrease in band intensity with increasing concentrations of Myc NTD. By comparison, clone 6-11-1 appeared to be relatively resistant to competition. Taken together, the results shown in Figure 8D and 8E indicated that specific binding between the Myc NTD and library proteins could be detected *in vitro*.

The library ^{35}S-labeled proteins were then incubated with MB1 and MB2 deletion mutant GST-fusion proteins, GST-ΔMB1 and GST-ΔMB2, to test for dependence of these interactions on the presence of these sequences (Figure 8F). Initial inspection of the results found that, under these conditions, neither deletion mutant exhibited complete loss of binding to any of the three library proteins tested. However, when compared to the wildtype GST-Myc NTD, some subtle differences could be observed in relative amount of radioactivity precipitated by each Myc Box mutant. For example, clone 6-11-1 appeared to be less efficiently bound by GST-ΔMB1 and GST-ΔMB2 thus resulting in lighter bands as compared to that precipitated by wildtype GST-Myc NTD. By comparison, clone 9-11-1 appeared to be as efficiently bound by GST-Myc NTD wildtype or mutant proteins resulting in similar band intensities across the reactions. Binding of clone 11-26-1 to the GST-fusions resulted in three distinct levels of band intensities with the GST-ΔMB2 mutant displaying the greatest deficit in binding as compared to wildtype.

Myc NTD and full length GSK3A interact in RTA and in vivo

Of the proteins identified to interact with the Myc NTD by our library screen, one clone in particular was of immediate interest to us. Clone 9-11-1 was found to encode the kinase domain of the GSK3α protein that has been implicated to phosphorylate Myc at the NTD in vivo(Lutterbach and Hann, 1994; Pulverer *et al.*, 1994). Full-length human GSK3α was cloned in-frame with TUP1 to determine if it would be able to interact with the activator bait panel in a similar manner to clone 9-11-1, which encodes only the kinase domain. Coexpression of full length GSK3α with the wildtype Gal4-Myc NTD

bait revealed that it rescued growth to a similar extent as clone 9-11-1. Similar to clone 9-11-1, full-length GSK3α conferred growth with coexpressed with c-Myc and not with N-Myc or L-Myc NTD baits. However, full length GSK3α had slight growth with ΔMB2 mutant and the unrelated VP16 bait where as clone 9-11-1 did not. Interestingly, the ΔMB1 bait, which is deleted for GSK3α phosphorylation site, had no growth on +FOA. Overall, these results show that the isolated kinase domain and the full-length GSK3α proteins behave in a comparable manner, indicating that the RTA system was able to appropriately identify interaction domains. Moreover, the RTA implicates the Myc MB1 sequenced and the kinase domain of GSK3α as the important determinants of interaction between these two proteins.

Discussion

We have now established a high throughput screen for identifying novel Myc NTD-interacting proteins. We demonstrate that the novel RTA system was able to correctly differentiate between positive (α-tubulin, TBP and p107) and negative (pRB, p130 and pBDH empty vector) TUP1-fusion prey control interactions at the Myc NTD in a sensitive and specific manner. By determining the optimal parameters of specificity and sensitivity using a panel of known Myc-NTD binding proteins, then allowed us to more clearly interpret data generated by novel proteins isolated through screening of a cDNA library specially constructed for use in the RTA. We found that binding events captured by RTA could be recapitulated *in vitro*. These initial leads identified by RTA may represent physiologically relevant Myc NTD protein interactions. Indeed, the RTA identified an interaction between the Myc NTD and the kinase domain of GSK3α, which

has been implicated in phosphorylation of Myc at a conserved T58 residue in MB1. We show that the GSK3α kinase domain alone and full-length proteins produced similar results in the RTA, indicating that the system was able to identify Myc NTD-interacting domains found within the context of the intact protein.

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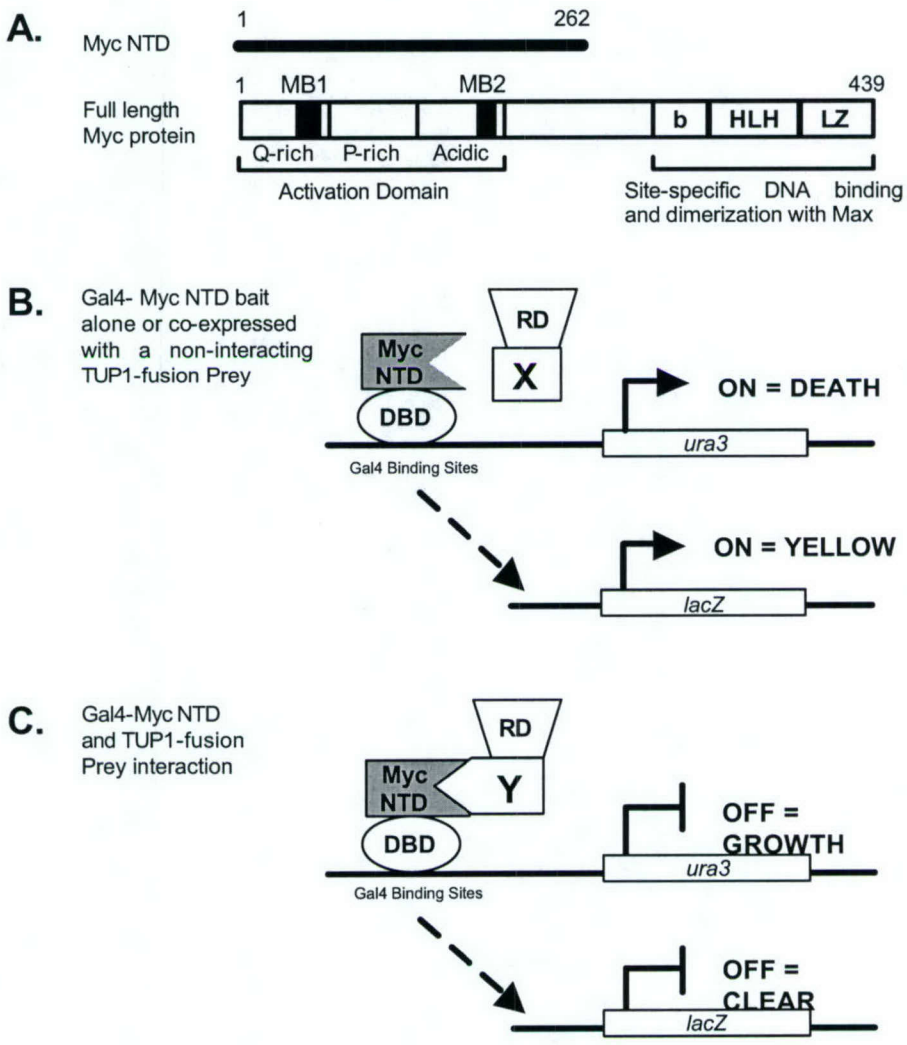

Figure 1 Outline of the basic components of the Myc RTA assay and reporter gene system
(A) Structure of the wildtype Myc protein. The CTD bHLHLZ motif is responsible for site-specific DNA binding and dimerziation of Myc with Max. The NTD activation domain can be characterized by three main areas: a glutamine-rich (aa 1-41), a proline-rich (aa 41-103) and an acidic region (aa 104-143). Two highly conserved Myc Box domains, MB1 (aa 45-63) and MB2 (aa 129-141) are also denoted. In this study the term "Myc NTD" will refer to amino acids 1-262 as denoted by the black bar. The Gal4-Myc NTD bait was constructed using the entire Myc NTD sequence and cloned as an in-frame fusion with the DNA-binding domain (DBD) of the yeast Gal4 protein **(B)** Schematic outline of the RTA and reporter gene system. The Mav108 reporter yeast strain contains two distinct reporter genes, *ura3* and *lacZ*. Expression of the Gal4-Myc NTD bait alone or co-expression with a non-interacting TUP1-fusion prey (X) allows for *ura3* reporter gene activation and results in death of yeast cells when plated on +FOA. Activation of *lacZ* can be detected by extent of ONPG substrate cleave and results in a yellow reaction solution. **(C)** Co-expression of the Gal4-Myc NTD bait with an interacting TUP1-fusion prey (Y) results in the rescue of yeast cell growth by suppression of the *ura3* reporter. Suppression of the *lacZ* reporter results in a relatively clear β-galatctosidase assay solution.

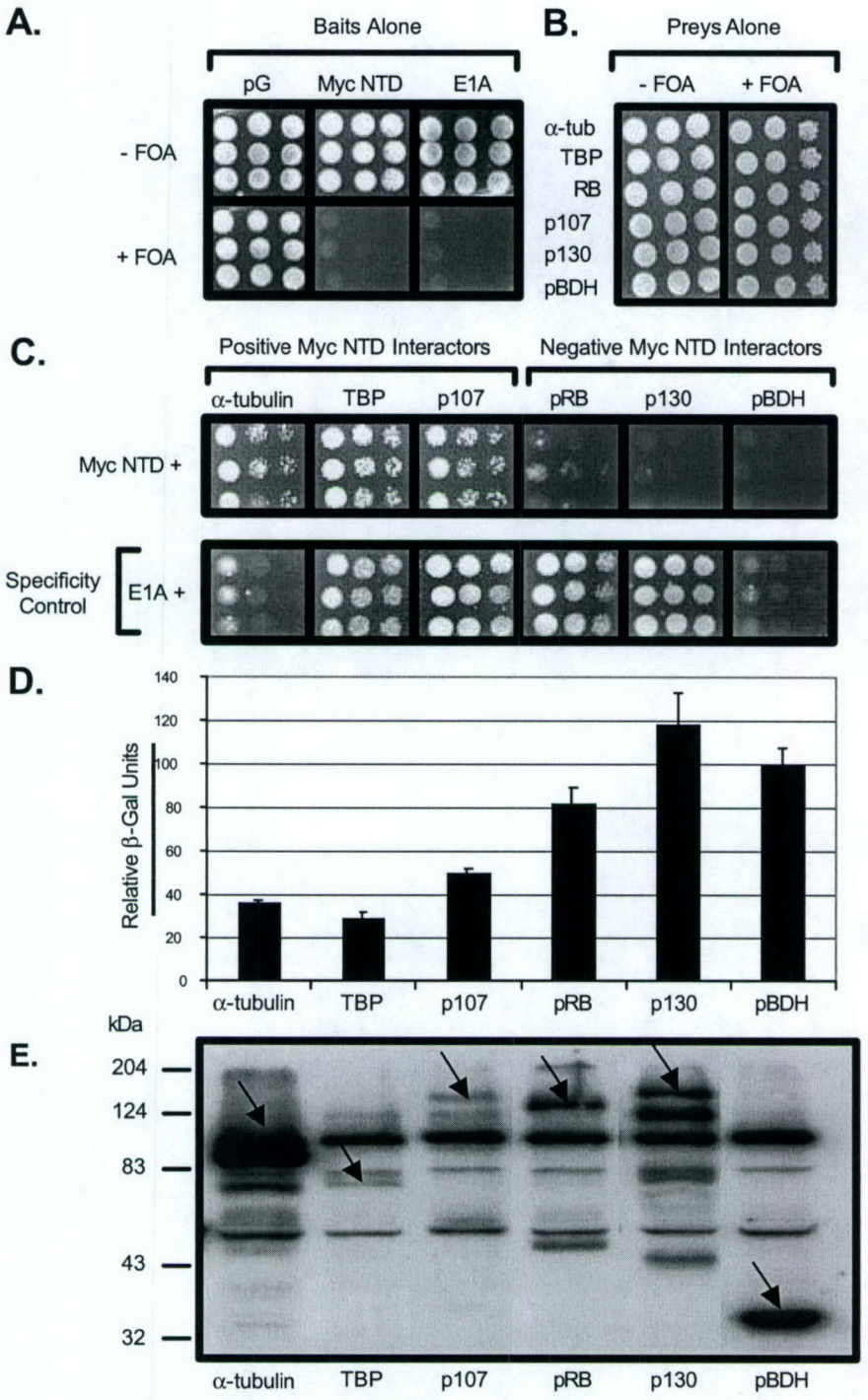

Figure 2 Control interactions for Gal4-Myc NTD and Gal4-E1A baits are detected by RTA using both reporters (A) Transformation of of pGT empty, Gal4-Myc NTD and Gal4-E1A bait vectprs alone into Mav108 reporter yeast strain. Gal4-Myc NTD and Gal4-E1A are able to activate URA3 reporter leading to death of yeast cell on FOA selection. Control cells plated onto FOA-free media or cell transformed with the non-activating pGT vector are able to grow. **(B)** Transformed yeast cells expressing of TUP1-fusion preys alone are able to grow on +/- FOA media. **(C)** A representative FOA plating of Gal4-Myc NTD and Gal4-E1A baits cotransformed with control prey panel. Positive control TUP1-fusion preys, α-tubulin, TBP and p107 are able to rescue growth of yeast expressing the Gal4-Myc NTD on FOA. Negative control TUP1-fusion preys, pRB, p130 and empty vector do not confer significant growth on FOA. Growth of Gal4-E1A expressing yeast cells was rescued by TBP and pRB-family TUP1-fusion proteins. All cotransformants were able to grow when plated onto FOA-free media (data not shown) **(D)** A representative liquid β-galactosidase assay for Gal4-Myc NTD cotransformants. **(E)** Expression of TUP1-fusion proteins in Mav108 yeast. Cotransformants were grown in liquid media and harvested in log phase for protein lysates. Lysates were electorphoretically separated, transferred to PVDF membrane and immunoblotted using a αTUP monoclonal antibody. Specific TUP1-fusion protein bands are marked by an arrow.

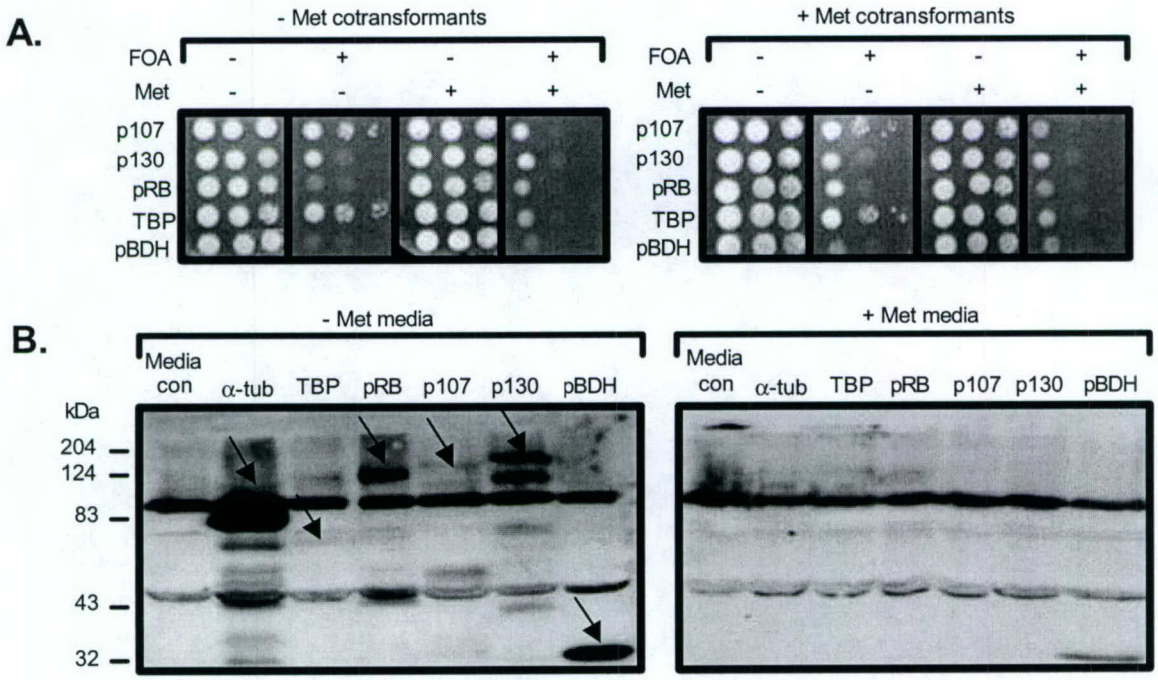

Figure 3 Rescue of growth on FOA is dependent on the concurrent expression of the interacting TUP1-fusion
(A) Representative plating of -Met and +Met grown cotransformants on FOA. Under +FOA-Met conditions, TUP1-fusion protein are expressed and positive control TUP1-fusions are able to rescue yeast cell growth indicating that an interaction with the Gal4-Myc NTD bait. By contrast, plating of the same cells onto +FOA+Met media results in loss of yeast cell growth indicating that the rescue seen on the matching +FOA plate is dependent upon the concurrent expression of the interacting TUP1-fusion prey. FOA Plating of +Met grown cotransformation shows that expression of TUP1-fusion preys can be efficiently reactivated and confers a similar level of rescue when compared to -Met grown cells **(B)** Immunoblot analysis using a αTUP1 monoclonal antibody indicates that TUP1-fusion prey protein expression, which can be detected under -Met (specific bands are denoted by an arrow), is efficiently suppressed when cells are grown in +Met media. -Met and +Met samples were included as the first lane of each blot as a control for background under each growth condition.

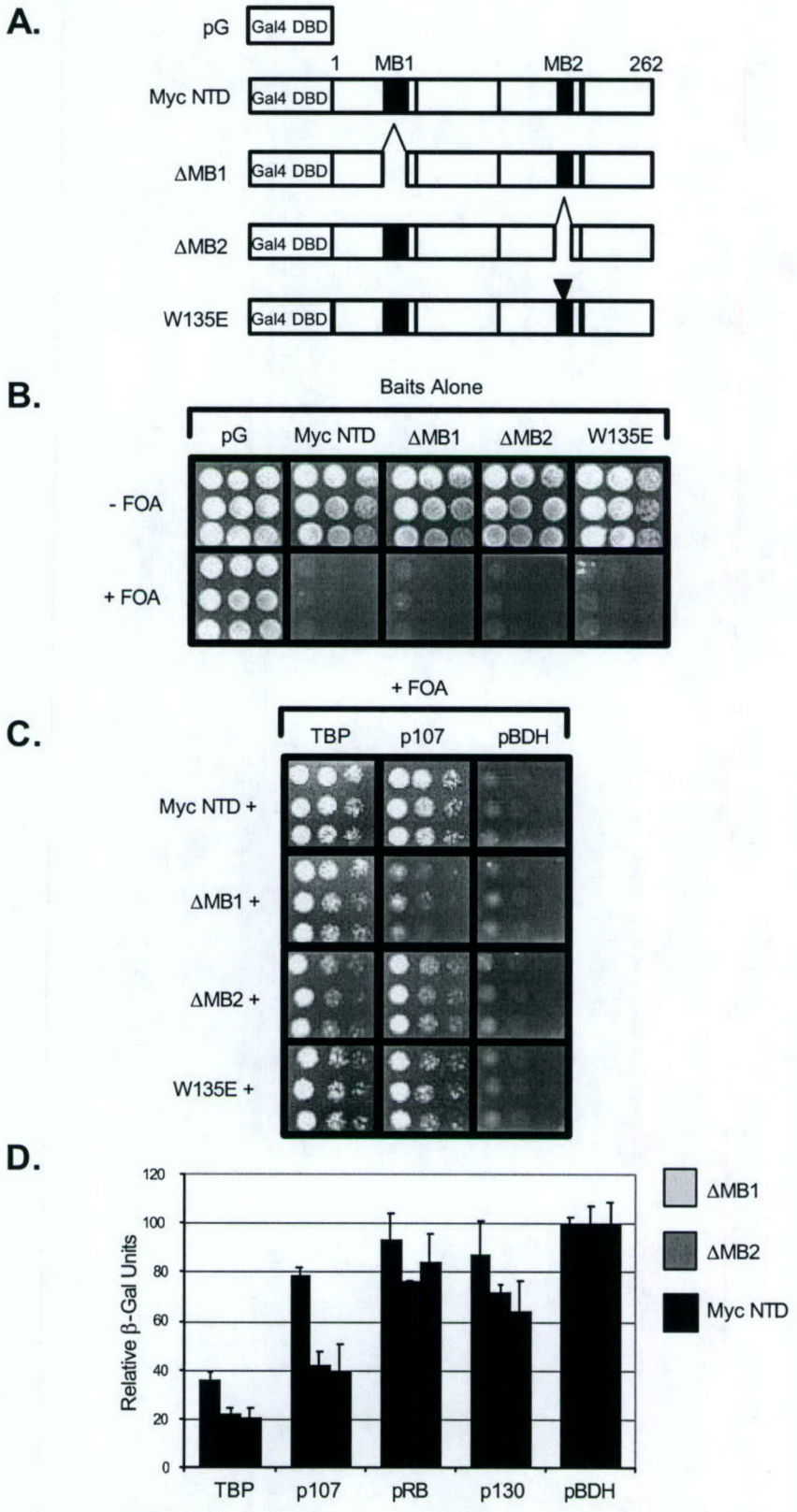

Figure 4 Differential binding of positive control TUP1-fusion preys to mutant Myc NTD baits
(A) Schematic outline of Gal4-Myc NTD mutant baits used in this experiment. **(B)** Plating of Myc mutant baits alone. Deletion of MB1 or MB2 and mutation at W135E does not affect ability of the Myc NTD to activate reporter *ura3* expression **(C)** A representative FOA plating of Myc NTD mutant baits cotransformed with TBP and p107 control TUP1-fusion preys **(D)** A representative liquid β-galactosidase assay.

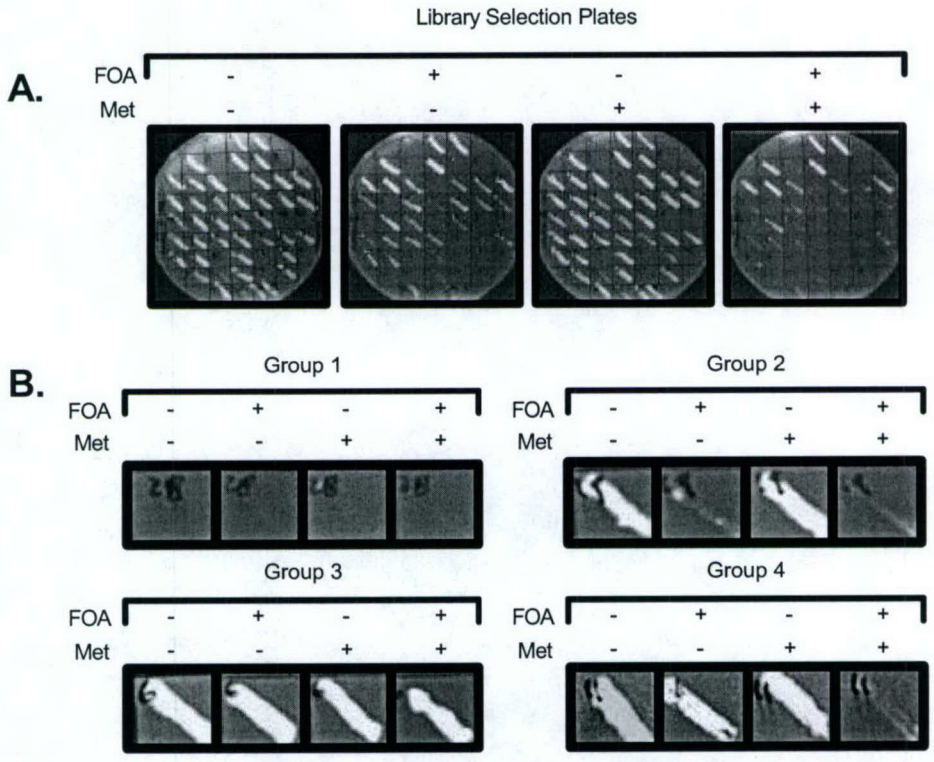

Figure 5 Identification of novel Myc NTD-interacting protiens by library screen using RTA
(A) A representative set of library selection plates. **(B)** Clones can be divided into 4 categories. Group 1 clones were viable when picked from original screening plates onto master plates, but were not viable when transferred from master plates to selection plates. Group 2 clones were viable on selection plates, but did not confer growth on FOA. Group 1 and 2 clones were not carried further. Group 3 clones were able to confer strong rescue on FOA, however, strong growth was also seen on +Met, suggesting that the growth on FOA could be non-specific. Group 4 clones exhibit the ideal selection criteria, rescue of growth on FOA and loss of this rescue when transferred to +Met indicating specificity of growth on FOA to the concurrent expression of the library TUP1-fusion.

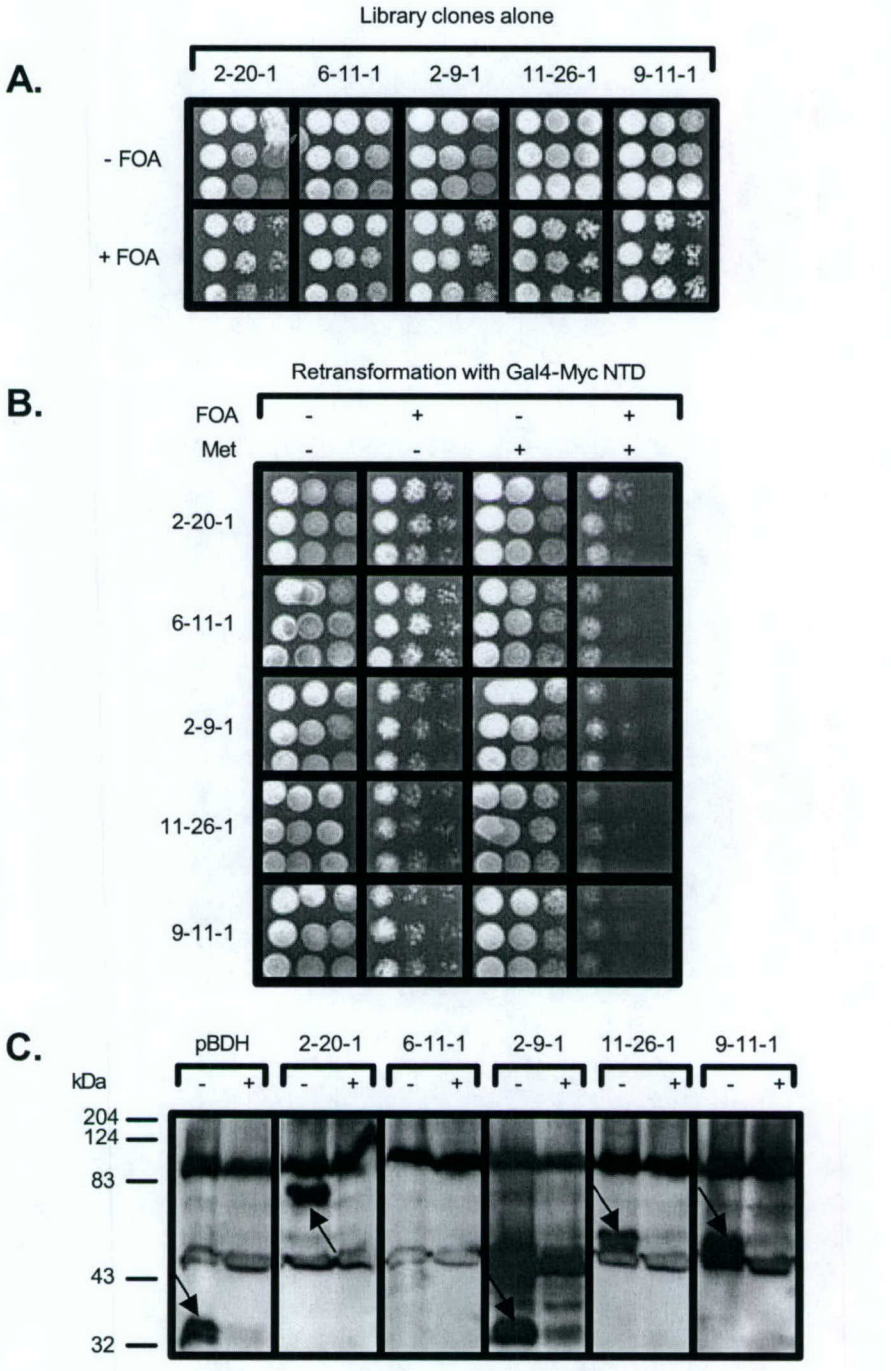

Figure 6 Library clone retransformation test and +/-Met protein expression analysis
(A) Expression of library clones alone on +/-FOA selection. **(B)** A representative panel of retransformation tests with Gal4-Myc NTD are shown. **(C)** Immunoblot analysis with an αTUP1 monoclonal antibody shows that under high methionine growth condition (+Met), expression of the specific TUP1-fusion library protein, denoted by an arrow, is significantly inhibited.

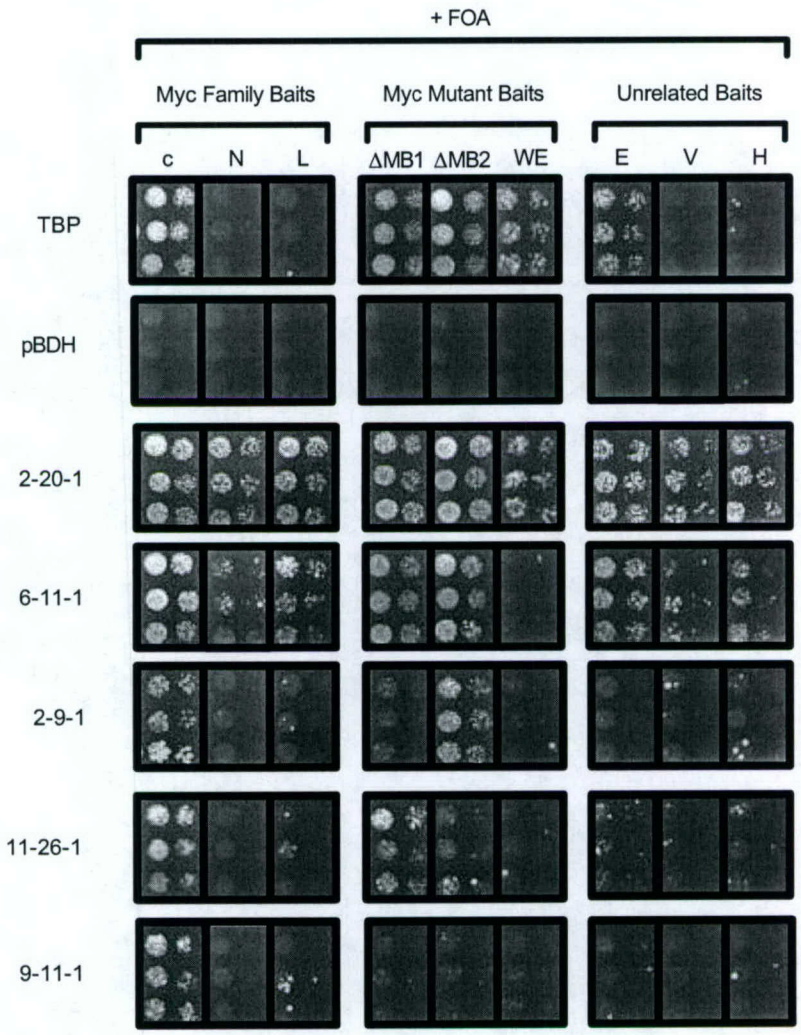

Figure 7 Activator panel test: Test of library clones for interaction with a panel of Myc-specific and non-specific activator baits

The activator panel of baits include NMyc and LMyc, to determine if TUP1-fusion library clones are able to interact with other members of the Myc family. Mutant Myc Box deletion baits, ΔMB1 and ΔMB2, and the W135E point mutation bait comprised the Myc mutant bait panel. Several unrelated activator baits, including full length E1A and the activation domains of VP16 and HSF1, were constructed to be used as controls for specificity of interaction with Myc proteins. A representative panel of +FOA results for 5 library clones are shown. Testing of the TBP positive control and pBDH negative control were also performed and shown for comparison. For simplicity, only the 1/10 and 1/100 dilutions for each bait and library clone combination are shown.

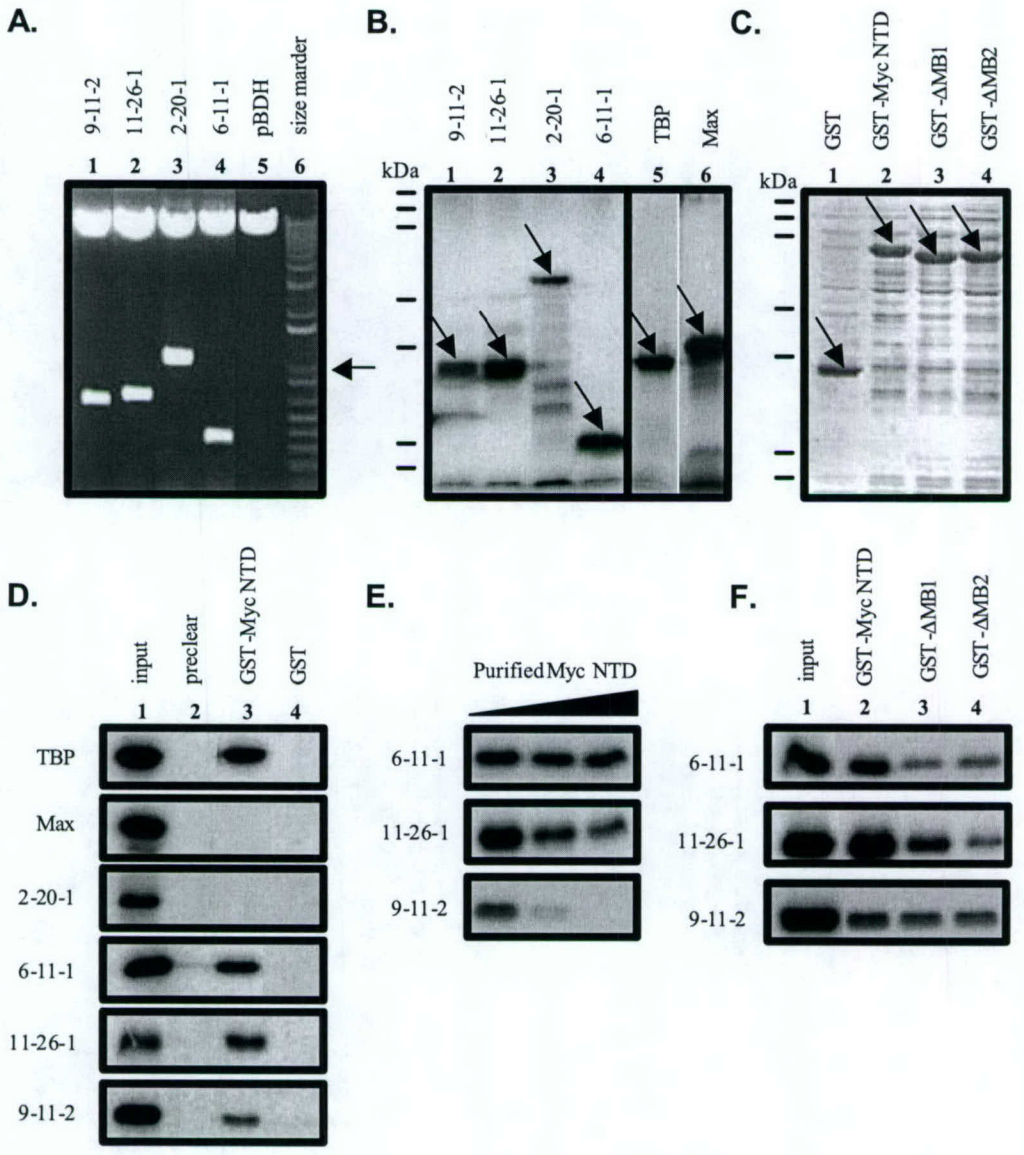

Figure 8 Examination of in vitro expression, binding and specificity for wildtype GST-Myc NTD and mutant GST-ΔMB1 and GST-ΔMB2 fusions with ^{35}S-methionine labeled library proteins

(A) Estimation of library cDNA insert size. SalI restriction enzyme digestion of TUP1-fusion encoding library vectors to release cDNA insert. The 1 kb migration on size standard is denoted by the arrow. **(B)** In vitro expression of library proteins using radiolabled ^{35}S-methionine. Electrophoretic separation and autoradiography revealed that each library clone was appropriately translated and expressed to adequate levels for use in in vitro experiments. Arrows denote specific bands corresponding to the translated product. **(C)** Recombinant expression of GST-fusion proteins cloned from wildtype Myc NTD (GST-Myc NTD) and Myc Box deletion mutant templates (GST- ΔMB1 and GST- ΔMB2). Electrophoretic separation of crude lysates and subsequent coomassie staining indicated the relative abundance of each GST-fusion protein. GST-fusion proteins denoted by an arrow **(D)** In vitro binding experiment to test for specificity of interaction with the wildtype GST-Myc NTD and control GST alone lysates. **(E)** In vitro competition experiment. Purified GST-free Myc NTD was added to binding reactions that were set-up as in (D). Results are shown for three concentrations of purified Myc NTD in increasing order from left to right as 1/10, equimolar and 4x molar ratios of GST-Myc NTD. Equalent amounts of GST-fusion protein and radiolabled library proteins were added to each reaction. **(F)** In vitro interaction potential of wildtype GST-Myc NTD and Myc Box deletion mutants GST-ΔMB1 and GST-ΔMB2 proteins with labeled library proteins.

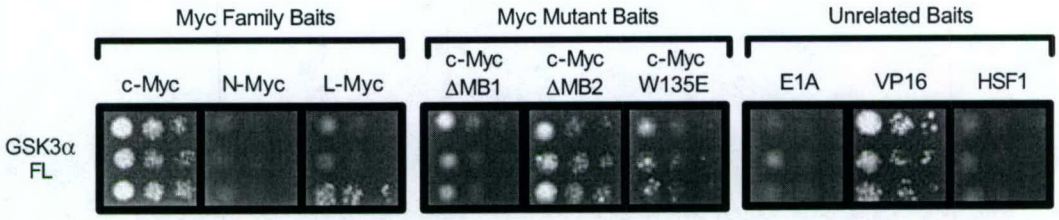

Figure 9 Full length human GSK3α and β TUP1-fusion protein binding profile with activator panel baits
Full-length (FL) human GSK3α was cloned as a TUP1-fusion and tested in RTA by coexpression with the activator panel previously used and described in Figure 7.